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Preparation of a standardized, efficacious agricultural H5N3 vaccine by reverse genetics

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Abstract

Options for the control of emerging and reemerging H5N1 influenza viruses include improvements in biosecurity and the use of inactivated vaccines. Commercially available H5N2 influenza vaccine prevents disease signs and reduces virus load but does not completely prevent virus shedding after challenge with H5N1 virus. By using reverse genetics, we prepared an H5N3 vaccine whose hemagglutinin is 99.6% homologous to that of A/CK/HK/86.3/02 (H5N1). We used the internal genes of A/PR/8/34 and the H5 of A/Goose/HK/437.4/99 (H5N1) after deletion of basic amino acids from its connecting peptide region. The resulting virus was not lethal to chicken embryos and grew to high HA titers in eggs, allowing preparation of HA protein-standardized vaccine in unconcentrated allantoic fluid. The N3 neuraminidase, derived from A/Duck/Germany/1215/73 (H2N3), permitted discrimination between vaccinated and naturally infected birds. The virus construct failed to replicate in quail and chickens. Similar to parental A/PR/8/34 (H1N1), it replicated in mice and ferrets and spread to the brains of mice; therefore, it should not be used as a live-attenuated vaccine. The H5N3 vaccine, at doses of 1.2 μg HA, induced HI antibodies in chickens and prevented death, signs of disease, and markedly reduced virus shedding after challenge with A/CK/HK/86.3/02 (H5N1) but did not provide sterilizing immunity. Thus, reverse genetics allows the inexpensive preparation of standardized, efficacious H5N3 poultry vaccines that may also reduce the reemergence of H5N1 genotypes.

Keywords: Reverse genetics derived influenza H5N3 vaccines; Chickens; Efficacy; Safety testing

Introduction

The emergence in Hong Kong of H5N1 influenza viruses that are highly pathogenic in domestic poultry and in humans provided the first evidence of lethal disease caused by direct transmission of avian influenza viruses to humans (de Jong et al., 1997; Claas et al., 1998; Shortridge et al., 1998; Subbarao et al., 1998; Yuen et al., 1998). The potential evolution of an H5N1 virus capable of human-to-human transmission was prevented by the slaughter of all poultry in Hong Kong in December 1997; afterward, cases of human H5N1 infection in Hong Kong swiftly disappeared (Subbarao and Katz, 2000). In 2001, the reemergence of H5N1 viruses of multiple genotypes (Guan et al., 2002a) was once more controlled by the slaughter of poultry in Hong Kong; the removal of virus from poultry markets precluded potential transmission to humans. The further reemergence of H5N1 influenza viruses in poultry in Hong Kong in February and November 2002 (J.S. (Malik) Peiris, unpublished

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data) was controlled by the slaughter of H5N1-positive flocks and infected poultry stalls in markets.

The putative parents of the H5N1/97 virus continue to circulate in poultry in the region; these comprise A/Goose/Guangdong/1/96(H5N1)-like virus, which provided the hemagglutinin (HA) gene, and the counterparts of A/Quail/Hong Kong/G1/97 (H9N2) and A/Teal/Hong Kong/W312/97 (H6N1), which provided the seven other gene segments (Cauthen et al., 2000; Webster et al., 2002b; Guan et al., 1999; Chin et al., 2002). In view of the opportunities for reassortment provided by the live poultry markets, it is not surprising that highly pathogenic H5N1 strains have continued to emerge. Studies of the multiple genotypes of the extremely pathogenic H5N1/01 and H5N1/02 viruses (Guan et al., 2002b) illustrate the propensity of the H5 hemagglutinin gene of A/Goose/Guangdong/1/96 (H5N1)-like viruses to generate highly pathogenic phenotypes by acquiring a variety of gene segments that are compatible with it.

Strategies to attempt to control the emergence of highly pathogenic H5N1 viruses in Hong Kong poultry markets have included the following: (1) After emergence of an H5N1 influenza virus in 1997 all the poultry in Hong Kong were slaughtered. In 2001, only birds in the markets and birds due to go to the markets were slaughtered, as no farms in Hong Kong were shown to be infected (Sims et al., 2002a,b). In the February–March 2002 outbreaks, slaughter of poultry was confined to infected farms and live poultry markets. (2) Serological screening of each truckload of poultry sold from local farms or entering Hong Kong SAR (Sims et al., 2003a,b). (3) Removal of aquatic birds including ducks and geese from the live poultry markets in 1998 (Sims et al., 2003a,b). (4) Introduction of one "clean day" per month when all poultry markets are simultaneously empty and are cleaned (Sims et al., 2003a,b). (5) Quail was identified as the poultry species that continued to reintroduce H6N1 and H9N2 viruses into the Hong Kong poultry markets and were banned (Sims et al., 2003a,b). While these measures have been successful at removing the different H5N1 influenza virus genotypes from Hong Kong poultry markets, they have so far not prevented reemergence of H5N1 influenza viruses. After the third reintroduction of H5N1/02 influenza virus in Hong Kong, the decision was made to investigate the use of inactivated vaccine to reduce the H5N1 virus load and inhibit the further reemergence of H5N1 genotypes. Additional emphasis was also placed on improving sanitation and biosecurity on farms.

Inactivated vaccine was successfully used to control the H5N2 outbreak in Mexico in 1995 (Villard and Flores, 1997). We investigated the efficacy of commercially available H5N2 influenza vaccine against H5N1/02 influenza viruses from Hong Kong. We also investigated the use of reverse genetics to prepare an H5N3 vaccine that is inexpensive, simple to create, efficacious, and, most importantly, standardized for antigen content. Each of these goals was achieved, as described here. This article serves as proof of the principle that reverse genetics can provide inexpensive, standardized agricultural influenza vaccines.

Results

Efficacy of commercial H5N2 vaccines

The only commercially available inactivated H5 influenza vaccine is against A/CK/Mexico/232/94 (H5N2). The HA of this virus has an 89.3% amino acid sequence identity with the HA of A/Chicken/Hong Kong/86.3/02 (H5N1). Because the H5N2 influenza virus used in the commercial vaccine was not available to us, we used a virus isolated during the same outbreak [A/CK/Hidalgo/232/94 (H5N2)] in serologic studies. To evaluate the immune response after single and multiple doses of commercial inactivated H5N2 influenza vaccine, we vaccinated 8-day-old chickens with one or two doses. A single dose of A/CK/Mexico/232/94 (H5N2) vaccine induced higher hemaggtutination inhibition (HI) titers to A/CK/Hidalgo/232/94 (H5N2) (1:100) than to A/CK/HK/86.3/02 (H5N1) (1:20) (Table 1) that increased after challenge. After two doses of vaccine, higher levels of prechallenge antibodies were induced that did not increase after virus challenge.

To evaluate the efficacy of commercial H5N2 vaccine against A/CK/HK/86.3/02 (H5N1), we performed three experiments (Table 1). The first determined the efficacy of a single dose of vaccine followed by a modest challenge dose of ten 50% chicken lethal doses (CLD_{50}) of A/CK/HK/86.3/02 (H5N1). None of the 19 vaccinated chickens developed disease signs; in contrast, six of the nine unvaccinated controls developed disease signs and died within 2–3 days. Although the 19 vaccinated birds showed no disease signs, 6 birds shed low levels of virus in their feces, and 1 of 10 unvaccinated contact birds became infected but did not die.

In the second experiment, the birds received two doses of vaccine and developed higher levels of HI antibodies (Table 1); these birds were challenged more aggressively with 100 CLD_{50} of A/CK/HK/86.3/02 (H5N1) (Table 1). Despite their relatively high levels of HI antibodies (1/333), 1 of the 10 birds died 9 days after challenge, 3 shed virus in the trachea, and half (3/6) of the contact unvaccinated birds died. All unvaccinated challenged birds died within 2–3 days. The bird that died had the lowest HI titer to the challenge virus (1/80).

In a third experiment (Table 1), birds that had received two doses of vaccine were challenged under conditions more akin to field conditions by being exposed to dying birds that had been inoculated with 100 CLD_{50} of A/CK/HK/86.3/02 (H5N1). Under these conditions, none of the vaccinated birds developed disease signs or died, but one shed virus (from the trachea).

Thus commercial inactivated oil-emulsion A/CK/Mexico/232/94 (H5N2) provides protection against challenge with low doses of A/CK/HK/86.3/02 (H5N1) virus but did

Table 1
Efficacy of commercially available inactivated H5N2 vaccine (A/Chicken/Mexico/232/94) in chickens

Vaccination status	Challenge virus	Mean HI antibody titer				Dead/total (dpc)	Virus isolation (no. positive/total) 3 dpc	
		to CK/HK/86.3/02 (H5N1)		to CK/Hidalgo/232/94 (H5N2)				
		Prechallenge	Postchallenge	Prechallenge	Postchallenge		Trachea (titer)	Cloaca (titer)
				Experiment 1				
Vaccinated, 8 days old	10 CLD_{50} A/Ck/HK/86.3/02 (H5N1)	20 ± 5	53 ± 8	100 ± 15	150 ± 20	0/19	0/19	6/19 (< 1 log_{10}/ml)
Unvaccinated contacts	None					0/10	0/10	1/10 (< 1 log_{10}/ml)
Unvaccinated controls	10 CLD_{50} A/Ck/HK/86.3/02 (H5N1)					6/9 (2–3)	0/3	1/3
				Experiment 2				
Vaccinated 8 days old and boosted 28 days later	100 CLD_{50} A/Ck/HK/86.3/02 (H5N1)	333 ± 134	337 ± 80	391 ± 80	257 ± 56	1/10 (9)	3/10 (1–2.25 log_{10}/ml)	0/10
Unvaccinated contacts	None					3/6 (4)	3/6 (< 1–2.25 log_{10}/ml)	2/6
Unvaccinated controls	100 CLD_{50} A/CK/86.3/02 (H5N1)					9/9 (2–3)	ND (Dead)	ND (Dead)
				Experiment 3				
Vaccinated 8 days old and boosted 28 days later	Exposed to birds infected with 100 CLD_{50} A/CK/HK/86.3/02 (H5N1)	282 ± 82	252 ± 72	388 ± 74	326 ± 76	0/10	1/10	0/10
Unvaccinated contacts	Exposed to birds infected with 100 CLD_{50} A/CK/HK/86.3/02 (H5N1)					3/4 (2)	0/1 (3 Dead)	0/1 (3 Dead)

dpc: days postchallenge; ND: not done.

not prevent virus shedding, which transmitted to contact birds. When the severity of challenge was increased in the second experiment, 1 of 10 vaccinated birds died but not in the third experiment. However, virus shedding in vaccinates in the first experiment was sufficient to infect 50% of unvaccinated contact controls. Despite high levels of HI antibody, the heavily challenged birds were not fully protected, thus raising the question of lack of genetic similarity between the vaccine and challenge virus.

Description of H5N3 vaccine derived by reverse genetics

The commercially available inactivated H5 influenza vaccines for poultry contain H5N2 virus from 1994 and are only 89.3% homologous at the amino acid level with H5N1/02 viruses from Hong Kong. Therefore, we used reverse genetics to make a high-yield, nonpathogenic H5N3 virus. We used the egg-adapted, high-growth influenza A/Puerto Rico/8/34 (H1N1) virus (PR8) as the donor of the internal genes, which had previously been cloned and rescued by reverse genetics (Hoffmann et al., 2002). We used the modified H5 hemagglutinin gene of A/Goose/Hong Kong/437-4/99 (H5N1) virus. This H5 hemagglutinin gene is antigenically closely related to the HA gene of A/CK/HK/86.3/02 (H5N1). To prevent the generation of a highly pathogenic virus, we deleted the nucleotides encoding four basic amino acids at the HA1–HA2 cleavage site, creating a cleavage site resembling that of the nonpathogenic H6 HA gene of influenza A/Teal/Hong Kong/W312/97 (Fig. 1). The N3 neuraminidase gene was obtained from the A/Duck/Germany/1215/73 (H2N3) virus. The N3 gene was chosen to generate a virus that can be used as a vaccine but that also allowed us to distinguish vaccinated birds from infected birds by detection of antibodies to N3.

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Wild-type:
CCT CAA AGA GAG AGA AGA AGA AAA AAG AGA GGA CTA TTT
Pro Gln Arg Glu Arg Arg Arg Lys Lys Arg*Gly Leu Phe

Modified H5 sequence:
CCT CAA ATT GAG ACA AGA --- --- --- --- GGA CTA TTT
Pro Gln Ile Glu Thr Arg --- --- --- ---*Gly Leu Phe
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Fig. 1. Modified A/Goose/HK/437-4/99 (H5N1) hemagglutinin sequence. Twelve nucleotides in the wild-type cDNA were deleted by genetic engineering to generate a recombinant H5-virus in which the basic amino acids (Arg Lys Lys Arg) flanking the cleavage site (represented by*) were deleted.

Table 2
Properties of parental and reverse genetics-derived viruses

Virus strain	HA titer	Infectivity titer (EID_{50})	Embryo death (CLD_{50})	H5 protein (μg/ml)	Plaque number in MDCK	
					+ Trypsin	− Trypsin
A/PR/8/34 (H1N1)	2048	8.38	No	NA	2.1×10^7	0
A/Goose/HK/437.4/99 (H5N1)	512	8.16	Yes (8.16)	NA	3.9×10^5	2.8×10^5
A/Duck/Germany/1215/73 (H2N3)	256	9.63	No	NA	2.9×10^6	0
rgH5N3-unmodified	2048	8.50	No	11.8	5.1×10^6	0
rgH5N3-allantoic fluid vaccine	640			11.8	NA	NA
rgH5N3-Amicon vaccine	20,480			75.9	NA	NA
rgH5N3-purified vaccine	102,400			5500	NA	NA

NA = not applicable.

Properties of parental and reverse genetics derived viruses

In vitro properties

The reverse genetics derived H5N3 (rgH5N3) viruses were characterized by their growth potential and plaque-forming ability in chicken embryos and in tissue culture (Table 2). The rgH5N3 virus grew to the same HA titer as the parental PR8 virus and thus has the desirable property of producing high yields of HA protein. With its modified HA, the rgH5N3 virus failed to kill chicken embryos or to produce plaques in tissue culture in the absence of trypsin, whereas the parental A/Goose/HK/437-4/99 (H5N1) virus killed chicken embryos and produced plaques in MDCK cells in the presence and absence of trypsin. The other parental viruses [PR8 (H1N1), A/Duck/Germany/1215/73 (H2N3)] and the rgH5N3 virus produced plaques only in the presence of trypsin. Therefore, the rgH5N3 virus has the following characteristics of a nonpathogenic virus: the absence of polybasic amino acids at the connecting peptide region of the HA, the inability to kill chicken embryos, and the failure to form plaques in tissue culture without trypsin.

In vivo properties

To determine the pathogenicity of the parental and reassortant viruses, we inoculated chickens, quail, mice, and ferrets. The rgH5N3 virus did not replicate in quail and chickens and was nonpathogenic, whereas the A/Gs/HK/437-4/99 (H5N1) was highly pathogenic in chickens (Table 3, see B). In mice, the rgH5N3 was less pathogenic than the parental PR8 or rgPR8 viruses. On day 3 after inoculation, pulmonary virus titers were similarly high in mice inoculated with rgH5N3, the parental PR8, or rgPR8. By day 7, the parental and rgPR8 viruses had killed the mice, whereas the rgH5N3 had not, although it was detectable at high titers in the lungs (6.2 $\log_{10}$ EID_{50}). Both the parental PR8 and the rgH5N3 viruses were detected in the brains of mice on day 3 or 4 after inoculation, although titers of parental PR8 were higher. By day 7 postinoculation, the parental virus had killed all inoculated mice, whereas the rgH5N3 had not, and no rgH5N3 virus was detected in the brain. The parental A/Goose/HK/437-4/99 (H5N1) and A/Duck/Germany/1215/73 (H2N3) viruses showed limited replication in mice (Table 3 see A).

In ferrets, the rgH5N3 viruses were detected in nasal washes on days 3 and 5 postinoculation, but at lower titers than the parental PR8 or rgPR8 viruses. The parental A/Goose/HK/437-4/99 (H5N1) replicated poorly in ferrets, whereas the A/Duck/Germany/1215/73 (H2N3) was detected at modest titers in nasal washes for up to 7 days. Daily measurement of temperature and observation revealed no disease signs in any of the ferrets; all animals gained weight and developed antibodies to the inoculated viruses.

Overall, the rgH5N3 virus was completely nonpathogenic in chickens and quail, and there was no evidence of virus replication. In ferrets and mice, the rgH5N3 virus was less pathogenic than the parental viruses.

Stability of rgH5N3 virus on passage

The rgH5N3 virus was passaged multiple times in chicken embryos to determine its genetic stability. At each passage, the eggs were examined for evidence of embryo death and for HA titer. After 14 passages, the HA gene was sequenced and the virus was tested antigenically with postinfection chicken antiserum. There was no evidence that the virus reverted to virulence in these 14 passages: there were no dead eggs; the HA sequence remained the same (Fig. 1); the pathogenicity did not change, and the virus retained its high growth characteristics (results not shown).

Yield of hemagglutinin protein from rgH5N3 virus

Although HA titers measure the ability of a virus to agglutinate erythrocytes, they do not accurately quantify HA protein content. We used single radial diffusion assays, which are conventionally used to standardize human influenza vaccine, to estimate the yield of HA protein from the rgH5N3 reassortant, and to standardize the vaccine for use in efficacy studies in chickens (Table 2). After inactivation of virus in allantoic fluid, the HA titer fell by approximately 75%, but the antigen content, as measured by single radial diffusion, remained constant.

Table 3
Animal testing of rg H5N3 vaccine and parental viruses

Virus	Animal	Route of inoculation	Pathogenicity[a]	Dose of inoculum (log_{10} EID_{50})	Virus titer (log_{10} EID_{50}) Lung or nasal wash			Brain		SeroConversion HI Antibody Titer	
					D 3/4	D 5	D 7	D 3/4	D 7	D0	D 11/14
A. In mice and ferrets											
A/PR/8/34	Mice	IN	1.9	6.2	7.0	NA	—[b]	3.3	—[b]	NA	NA
	Ferrets	IN	None	7.2	5.3	3.3	<[d]	NA	NA	< 40	20,480
A/Goose/HK/437-4/99	Mice	IN	—[f]	6.8	3.2	NA	1.5	<	NA	NA	NA
	Ferrets	IN	None	5.8	1.4	<	<	NA	NA	< 40	80–160
A/Duck/Germany/1215/73	Mice	IN	6.9	6.2	<	NA	<	<	<	NA	NA
	Ferrets	IN	None	7.2	3.2[e]	2.1	1.9[e]	NA	NA	< 40	160
rgH5N3	Mice	IN	6.0	5.5	7.7	NA	6.2	1.8	<	NA	NA
	Ferrets	IN	None	6.5	3.2	2.3	<	NA	NA	< 40	160
rgPR8	Mice	IN	3.3	7.3	8.5	NA	–[b]	<	–[b]	NA	NA
	Ferrets	IN	None	7.6	4.5	4.6	<	NA	NA	< 40	20,480

Virus	Animal	Route of inoculation	Dose of inoculum ($log_{10}EID_{50}$)	Virus isolation on day 2, 4, or 6 p.i.		Disease signs/dead/total
				Trachea	Cloacal	
B. In quail and chicken						
A/Goose/Hong Kong/437-4/99	Chicken	IV	6.1	8/8	8/8	8/7/8
rgH5N3	Quail	IN, IO, IT	7.2	0/4	0/4	0/0/4
	Chicken	IN, IO, IT	7.5	0/6	0/6	0/0/6
	Chicken	IV	6.8	0/8[c]	0/8	0/0/8

IN, Intranasal; IV, intravenous; IO, intraocular; IT, intratracheal.
[a] $Log_{10}LD_{50}$ values are shown as the number of $log_{10}EID_{50}$ resulting in 50% mortality.
[b] All mice died.
[c] Chickens swabbed on day 3 only.
[d] <, no virus detected.
[e] The average EID_{50} from two ferrets; no virus was detected in one animal.
[f] Pathogenicity was determine by inoculation with $10^{6.8}$ EID_{50} and resulted in 50% mortality ($n = 8$).
d: day after inoculation; NA: not applicable.

Efficacy of the rgH5N3 vaccines

To determine the efficacy of rgH5N3 vaccines against highly pathogenic A/CK/HK/86.3/02 (H5N1) virus in chickens, we used three experiments to answer different questions.

The first experiment investigated whether the H5N3 vaccine must be concentrated and purified to yield sufficient antigen to induce protective immunity against the highly pathogenic A/CK/HK/86.3/02 (H5N1) strain in chickens. Groups of five chickens were vaccinated with standardized doses of allantoic fluid containing rgH5N3 virus, with 10-fold Amicon-concentrated allantoic fluid containing rgH5N3 virus, or with concentrated and purified rgH5N3 virus. Each of the H5N3 vaccine preparations induced similar high levels of HI antibody to both A/Gs/HK/437-4/99 (H5N1) and A/CK/HK/86.3/02 (H5N1), showing the close antigenic similarity of these viruses (Table 4). The mean HI

Table 4
H1 responses induced by allantoic-fluid, Amicon-concentrated, and purified rgH5N3 vaccines in chickens (Experiment 1)

Vaccine	HA antigen dose (μg)	HI titer to Ck/HK/86.3/02 (H5N1) before challenge	HI titer to Gs/HK/437-4/99 (H5N1) before challenge
Allantoic fluid oil-emulsion	1.2	194 (10–320)	196 (100–320)
Amicon-concentrated (10×) oil-emulsion	7.6	168 (80–640)	184 (80–640)
Amicon-concentrated (10×) 1:10 diluted oil-emulsion	0.76	192 (80–320)	192 (80–320)
Purified 1:31.6 diluted oil-emulsion	17.4	169 (80–320)	169 (80–320)
Purified 1:100 diluted oil-emulsion	5.5	256 (160–320)	256 (160–320)
Control	0	0	0

Table 5
Efficacy of allantoic-fluid, Amicon-concentrated, and purified rgH5N3 vaccines against naturally transmitted H5N1/02 virus

Vaccine	HA antigen dose (μg)	Dead/ total	Virus shedding/ total on day 4	Mean HI titer to A/Ck/HK/86.3/02 (H5N1) pre/postchallenge
Experiment 2				
Allantoic fluid oil-emulsion	1.2	0/9	2/9	ND/133 (40–320)
Amicon-concentrated (10×) 1:3.16 diluted oil-emulsion	2.4	0/9	1/9	ND/133 (80–330)
Purified, 1:100 diluted oil-emulsion	5.5	0/9	0/9	ND/226 (40–640)
Purified 1:100 Freund's adjuvant	5.5	0/9	0/9	40/67 (40–160)
Control		8/9	5/6	
Experiment 3				
Allantoic fluid oil-emulsion vaccine	1.2	0/26	1/26	15.8/110 (0–640)
Allantoic fluid 1:10 diluted oil-emulsion vaccine	0.12	6/9	4/9	0/33.3 (0–80)
PBS control		26/27	22/27	0/(0–20)

titer induced by the allantoic fluid vaccine (1:194) was as high as those induced by the Amicon-concentrated vaccine (1:168) or purified vaccine (1:192), and higher doses of purified H5N3 antigens did not induce higher HI titers than unconcentrated allantoic fluid vaccine.

In the second experiment (Table 5), the same allantoic fluid, Amicon-concentrated, and purified vaccines were given to groups of nine chickens. An additional group of chickens was vaccinated with purified vaccine in Freund's incomplete adjuvant to determine the comparative efficacy of the oil-emulsion vaccine. Three weeks later, one chicken infected with 100 CLD_{50} of A/CK/HK/86.3/02 (H5N1) was introduced into each group of nine vaccinated birds to mimic field conditions of exposure. None of the groups of vaccinated chickens (Table 5) developed signs of disease, and none of the birds died. However, two birds that received the allantoic fluid vaccine and one that received the Amicon-concentrated vaccine shed virus at very low levels (detectable only in undiluted samples). In comparison, all nine of the control unvaccinated birds developed signs of highly pathogenic influenza infection, and eight died. It is noteworthy that the Freund's adjuvant vaccine did not induce antibody titers as high as those induced by the oil-emulsion vaccine mean (HI titer = 1:67 for Freund's vaccine group versus 1:190 for the oil emulsion group) and both groups provided complete protection with no virus shedding after challenge.

The third experiment was conducted to determine the dose response to the allantoic fluid vaccine and to determine how often virus shedding was detected in vaccinated birds (Table 5). The allantoic fluid vaccine containing 1.2 μg/dose of HA protein was very efficacious, with disease signs or death observed in 0/26 birds; only 1/26 shed virus (very low levels, detectable only in undiluted samples). When the allantoic fluid vaccine was diluted 10-fold (0.12 μg/dose), no HI antibodies were detected in prechallenge sera from vaccinated birds; all birds shed virus, and 6/9 died.

Overall, these results show that the allantoic fluid of the H5N3 vaccine in oil-emulsion adjuvant is efficacious against A/CK/HK/86.3/02 (H5N1) influenza virus without concentration or purification. A single dose of vaccine containing 1.2 μg of H5 antigen prevents disease signs and death and reduces virus shedding to very low titers.

Induction of neuraminidase-inhibiting antibodies

The rgH5N3 vaccine was designed to allow distinction between birds infected in the field with H5N1 virus and those vaccinated with H5N3 vaccine. Neuraminidase inhibition (NI) titrations revealed antibodies to N3 in all sera that contained HI antibodies to H5. Birds vaccinated with 1.2 μg of rgH5N3 vaccine had a mean prechallenge titer of 1:67 to N3 and 1:27 to H5.

Discussion

The persistent reemergence of H5N1 influenza viruses in poultry in southeast China is of continuing concern to medical and veterinary public health officials. Although we know that the H5N1/97 was transmitted to 18 hospitalized persons and killed 6, we do not know the capacity of the H5N1 genotypes isolated from poultry in Hong Kong in 2001 and 2002 to infect humans. Experiments in chickens and quail show that these viruses are highly pathogenic (Guan et al., 2002a), and they are neurovirulent in mice without adaptation (Lipatov et al., 2003). Therefore, the 2001 and 2002 H5N1 viruses may have the potential to infect humans. The very recent detection of H5N1 virus in two humans in Hong Kong (February 2003) underscores these concerns.

Despite multiple strategies used to inhibit the reemergence of H5N1 influenza viruses in Hong Kong poultry markets (reviewed in the Introduction), nothing has so far prevented the reemergence of different genotypes of H5N1 viruses. After May 2002, inactivated A/CK/Mexico/232/94 (H5N2) oil emulsion vaccine was used at 22 farms near the 4 farms in the New Territories of Hong Kong that had had outbreaks in early 2002. No outbreaks of H5N1 infection or disease has occurred at these 22 farms, despite recent out-

breaks (December 2002–January 2003) in two waterfowl parks and five local chicken farms, and despite concurrent lethal H5N1 virus infections in wild ardeid birds (little egrets and grey herons) and waterbirds (black-headed gulls, flamingos, geese, swans, and ducks) in Hong Kong. Therefore, the A/CK/Mexico/232/94 (H5N2) is an efficacious vaccine against H5N1 viruses under field conditions in Hong Kong, despite antigenic drift in the hemagglutinin.

Inactivated influenza vaccine to highly pathogenic influenza viruses is a viable option for control strategies (Swayne et al., 2000). H5N2 influenza outbreaks in domestic poultry in Mexico in 1994 were controlled by improving sanitation and biosecurity and by using inactivated vaccine (Villard and Flores, 1997). The use of vaccination without improved sanitation and biosecurity would be very unsound and could mask the presence of H5N1 virus. Mexico has been successful at using the combination of improved biosecurity and vaccination to eradicate highly pathogenic influenza viruses from poultry.

Our findings show that both commercially available H5N2 vaccine and reverse genetics prepared H5N3 vaccine are efficacious at preventing signs of infection by highly pathogenic H5N1 viruses and at reducing virus shedding and spread to contact birds. The advantage of the H5N2-inactivated vaccine is that it is commercially available and has been used in large numbers of poultry. The disadvantage is that the commercial H5N2 vaccine is not standardized for antigen content, which may differ from batch to batch. H5N2 vaccines produced for use in Mexico were shown to vary substantially among batches; some batches were efficacious, while others, prepared by the same protocols, were not (Garcia et al., 1998). Although similar studies have not been done with the inactivated oil emulsion A/CK/Mexico/232/94 (H5N2) vaccine, the lack of standardization leaves such vaccines open to doubt. If vaccine standardization is improved, there will be lot-to-lot consistency, more certainty about vaccine efficacy, and the possibility of reduced vaccine cost, as antigen content can be better defined. Another problem in producing an inexpensive vaccine is the fact that available technologies do not ensure sufficient antigen content in unconcentrated allantoic fluid. The strategy of reverse genetics now permits the preparation of high-yield, efficacious virus vaccine for poultry that do not require concentration or purification.

Neither the commercial H5N2 vaccine nor the standardized reverse genetics H5N3 vaccine provided sterilizing immunity in chickens; low levels of virus shedding were detected in birds vaccinated with both types of vaccine. There is no doubt that H5 influenza vaccines can reduce clinical symptoms and virus spread, but without standardization of vaccine content, subpotent batches of vaccine will inevitably occur that may protect against clinical signs of disease and mask virus shedding. In Hong Kong all vaccinated farms have nonvaccinated sentinel birds to guard against this possibility.

While use of nonstandardized vaccines may not be a problem for avian influenza disease control, they may be a serious problem for human pandemic influenza preparedness. Such vaccines have the possibility of masking disease signs while the birds continue to shed viruses and persistence of virus infection in the presence of a flock immunity may contribute to increased virus evolution.

Our detection of parental PR8 and rgH5N3 influenza in mouse brain conflicts with the findings of Subbarao and colleagues (2003), who did not detect H5N1 made by reverse genetics in mouse brain. The viruses differed in their source of HA and NA, but both viruses derived their remaining genes from PR8 (H1N1). Strains of PR8 in different laboratories may have point mutations that cause them to differ in their characteristics. Hatta et al. (2001) showed that a mutation in PB2 (E627K) plus a cleavable HA is associated with mouse neurovirulence of the H5N1/97 viruses. Neither of these features is present in the H5N3 virus; the PB2 was derived from PR8 (H1N1), and the modified H5 HA does not possess a series of basic amino acids at the cleavage site. Because our rgH5N3 viruses are being used as inactivated preparations, potential neurovirulence presents no risk to vaccine recipients. On the other hand, these findings would be of concern if the vaccine were used as a live, attenuated preparation. Our results highlight the need to evaluate each influenza virus reassortant carefully, because our knowledge of neurotropism is incomplete.

The use of an inactivated influenza vaccine containing a marker NA (N3) permits discrimination between infected and vaccinated birds. In the present studies, antibodies to N3 were detected by NI tests, providing the proof-of-principle of this strategy. This approach was used previously by Capua et al. (2002) in the control of H7N1 avian influenza outbreaks in Italy. It remains to be determined whether influenza virus vaccines that contain homologous NA or homologous internal antigens (PB2, PB1, PA, NP, NA, M, NS) would be more efficacious. The available evidence indicates that the immunogenicity of HA is the most important factor in protecting against human infection (Wright and Webster, 2001), but immunity to NA and to all viral gene products provides both humoral and cell-mediated immune responses (Ada and Jones, 1986; Epstein et al., 1998; Kilbourne et al., 1995). The use of PR8 (H1N1) internal genes to provide high yields of HA protein and the use of N3 NA to allow discrimination between infection and vaccination may have reduced the potential efficacy of the vaccine, but these “costs” are balanced by the possibility of providing inexpensive, standardized influenza vaccines that can discriminate between infections and vaccination of poultry.

In summary, it has been demonstrated that reverse genetics provides a strategy for preparing high levels of antigen in chicken embryos that can be used to formulate standardized inexpensive vaccines that are efficacious against highly pathogenic H5N1 influenza viruses. Such vaccines may enhance control measures in SE Asia against continuing reemergence of highly pathogenic H5N1 avian

influenza viruses in poultry. The potential for these avian H5N1 viruses to spread to swine and humans is of continuing concern and methods for reducing the level of H5N1 viruses in the field in SE Asia are urgently needed. This will only be achieved by a combination of improved biosecurity, effective surveillance, and monitoring plus support from an effective vaccination program. In addition to providing the strategy for providing sufficient antigen for standardized vaccine, reverse genetics permits rapid changing of the vaccine strain when antigenic drift occurs.

Materials and methods

Viruses

Influenza viruses A/PR/8/34 (H1N1), A/Goose/Hong Kong/437-4/99 (H5N1), A/Duck/Germany/1215/73 (H2N3), A/Chicken/Hong Kong/86.3/02 (H5N1), and A/Chicken/Hidalgo/28159-232/94 (H5N2) were obtained from the repository of St. Jude Children's Research Hospital. The highly pathogenic H5N1 viruses were handled in BL3+ facilities at St. Jude Children's Research Hospital.

Reverse genetics

RT-PCR was performed with segment-specific primers as previously described (Hoffmann et al., 2001). Briefly, RNA was isolated by using the RNeasy kit (Qiagen) and transcribed to cDNA with the Uni12-primer (AGC AAA AGC AGG). Plasmids encoding the PB1, PB2, PA, NP, M, and NS genes of influenza PR8 were constructed as previously described (Hoffmann et al., 2002) and were designated pHW191-PB2, pHW192-PB1, pHW193-PA, pHW195-NP, pHW197-M, and pHW198-NS. The plasmid pHW251-HAΔ5, which encoded the HA of the A/Goose/HK/437-4/99 (H5N1) virus with a deletion of the polybasic amino acid region at the HA1–HA2 cleavage site, was derived by PCR amplification of two fragments of the plasmid pHW250-HA encoding the full-length H5. The primers used for the amplification of the two fragments have HA-specific regions (in bold) flanked by sequences for the type II restriction enzyme *Bsm*BI (underlined):

Bm-HA-1: 5′-TATTCGTCTCAGGG**AGCAAAAGCAGGGG**-3′
Bm-H5-1025R: 5′-ATTACGTCTCT**CCTCTTGTCTCAATTTGAGGGGTATT**-3′
Bm-H5-1020: 5′-ATTACGTCTCA**GAGGACTATTTGGGGCTATAGCAGG**-3′
Bm-NS-890R: 5′-ATATCGTCTCGTATT**AGTAGAAACAAGGGTGTTTT**-3′

The fragments were digested with *Bsm*BI and inserted into pHW2000-*Bsm*BI by a three-fragment ligation reaction. The N3 NA of influenza A/Duck/Germany/1215/73 was cloned into the pHW2000 vector by using PCR and the N3-specific primers previously described (Hoffmann et al., 2001). The Center for Biotechnology at St. Jude Children's Research Hospital determined the sequence of template DNA by using Rhodamine or dRhodamine dye-terminator cycle sequencing ready reaction kits with AmpliTaq DNA polymerase FS (Perkin–Elmer Applied Biosystems, Inc., Foster City, CA) and synthetic oligonucleotides. Samples were separated by electrophoresis and analyzed on PE/ABI model 373, model 373 Stretch, or model 377 DNA sequencers.

Generation of recombinant viruses

Recombinant viruses were generated by DNA transfection as described previously (Hoffmann et al., 2002). Briefly, 293T and MDCK cells were cocultured (0.2 to 1×10^6 cells of each cell line) and used for the transfection experiments. The cocultured cells were transfected with a DNA–lipid complex containing 1 μg of each plasmid, 18 μl of transit LT1 (Panvera, WI) in a final volume of 1 ml of OPTIMEM-I (Gibco, NY). Transfection was carried out for 6 h, at which time the DNA–lipid complexes were removed and replaced with fresh medium. The cells were incubated for an additional 24 h, and 0.5 μg/ml of TPCK-treated trypsin (Worthington) was added. After 72 h, the supematant was taken from the cells, and 100 μl was injected into 10-day-old embryonated chicken eggs. The allantoic fluid of this first egg passage stock was analyzed by HA assay and showed a titer of 2048 HA units/50 μl. The allantoic fluid (200 μl) was analyzed by RT-PCR, and each viral segment was partially sequenced to confirm the identity of the recombinant virus.

Preparation of vaccines

Viruses were propagated in the allantoic cavities of 10- to 11-day-old embryonated chicken eggs at 35°C for 48 h. Allantoic fluid was harvested, and virus was inactivated by adding β-propiolactone (BPL) at a ratio of 1:2000 (vol/vol) and allowing the fluid to remain at room temperature for 4 h and then remaining at 4°C for 24 h. Inactivation was confirmed by the absence of detectable infectivity after two blind passages of the treated allantoic fluid in embryonated eggs.

Virus concentration by Amicon ultrafiltration

Inactivated viruses in allantoic fluid were clarified by centrifugation at 5000 rpm for 15 min. The supernatant allantoic fluid was concentrated to 1/10 of its original volume by use of an Amicon concentrator ultrafiltration apparatus.

Virus purification

The concentrated viruses were purified by ultracentrifugation through a 25 and 70% sucrose cushion and then pelleted at 27,000 rpm at 4°C for 1 h. The pellet was

resuspended in STE buffer, sonicated for 2 min, and then centrifuged on a 25 to 70% sucrose continuous gradient with an SW28 rotor at 24,000 rpm for 2.5 h. Virus bands were removed by syringe, diluted in STE, and then pelleted as described above. The pellets were resuspended and sonicated in appropriate volumes of STE, and sodium azide was added at 200 ppm final concentration.

Standardization of vaccine

The content of the hemagglutinin protein in the allantoic fluid, Amicon-concentrated vaccine, and purified vaccine was standardized by the single radial immunodiffusion technique as described previously (Wood et al., 1985), using reagents specifically developed for H5N1/97 HA (Wood et al., 2001). The inactivated virus preparations, standardized in terms of micrograms of H5 HA protein, were emulsified in paraffin oil, which is currently used commercially to prepare influenza virus vaccines for poultry.

Commercial H5N2 influenza vaccine

A/Chicken/Mexico/232/94 (H5N2), chemically inactivated and emulsified in mineral oil to act as an adjuvant, was obtained from Intervet.

Biological testing of reverse genetics derived (rg) H5N3 vaccine

Coturnix quail

Adult quail (B&D Game Farm) were inoculated with 0.5 ml of a 1:10 dilution of rg-A/Goose/HK/437-4/99 (HA) × A/Duck/Germany/1215/73 (N3) with PR8 internal genes (rgH5N3) via the natural routes (intratracheally, intraocularly, and intranasally). The trachea and cloaca of each bird were swabbed on days 2, 4, and 6 postinoculation.

Specific pathogen-free (SPF) white leghorn chickens

Five-week-old chickens (Spafas, CT) were infected with 1.0 ml of a 1:10 dilution of the rgH5N3 virus via natural routes (intratracheal, intraocular, intranasal). Tracheal and cloacal samples were taken 2, 4, and 6 days postinoculation. Six-week-old white leghorn broiler chickens (Marshall Durbin) were intravenously inoculated with 0.2 ml of a 1:10 dilution of rgH5N3. Tracheal and cloacal swabs were taken 3 days postinoculation.

BALB/c mice

Female 8-week-old mice (The Jackson Laboratory, Bar Harbor, ME) weighing 18–20 g were used for virus pathogenicity and replication studies. Mice were anesthetized by inhalation of isolfluorane and inoculated intranasally with 0.1 ml of 10-fold virus dilutions. The mice were weighed and observed daily for 16 days for signs of disease and mortality. To determine virus titers in the lungs and brain, groups of mice were sacrificed on day 3 or 4 and on day 7, and virus titers in the tissues were determined by EID_{50} in 10-day-old embryonated chicken eggs.

Ferrets

Male ferrets 12 to 13 weeks old (Triple F Farms, Sayre, PA) were used to assess virus replication and pathogenicity. The animals were seronegative to the currently circulating human influenza A virus strains [A/New Caledonia/2007/99 (H1N1) and A/Panama/20/99 (H3N2)]. Ferrets were anesthetized with isofluorane and inoculated intranasally with 5.8, 6.5, or 7.6 EID_{50} of virus. Rectal temperatures and observations were made daily for 11–14 days for disease signs and mortality. On days 3, 5, and 7, ferrets were sedated with ketamine–HC1 (30 mg/kg), and 0.5 ml of PBS was inserted into each nostril, expelled onto a petri dish, and titrated in 10-day-old embryonated chicken eggs. Fourteen days after inoculation, the ferret sera were tested by HI for antibodies to the infecting virus.

Studies of commercial H5N2 vaccine

Forty SPF white leghorn chickens (Spafas) were vaccinated subcutaneously with a 0.5 ml volume at the base of the neck at 8 days of age. In the second experiment, 20 of the vaccinated chickens received booster doses of vaccine 4 weeks after the first vaccination by the same method and route. Three weeks after vaccination, chickens were moved to a BSL3+ animal housing facility and challenged with 10 CLD_{50} of A/CK/HK/86.3/02 (H5N1) in 0.3 ml given intranasally. Unvaccinated, uninfected contact chickens were placed in cages with vaccinated challenged birds 1 day later to detect transmission of the challenge virus from vaccinated birds. In addition, 10 unvaccinated chickens were infected with 10 CLD_{50} or 100 CLD_{50} of A/CK/HK/86.3/02 (H5N1) in 0.3 ml intranasally to serve as controls for the highly pathogenic virus. Chickens that were vaccinated and boosted were challenged 3 weeks postboost with 100 CLD_{50} of A/CK/HK/86.3/02 (H5N1) in 0.3 ml given intranasally. Three unvaccinated, uninfected chickens were placed in the same cage to serve as direct contact controls. In the third experiment, four unvaccinated chickens were inoculated with 100 CLD_{50} in 0.3 ml given intranasally and then placed in cages with vaccinated chickens to determine whether the vaccinated birds were protected from natural transmission of the highly pathogenic virus.

Blood was obtained from the wing veins of all chickens prior to challenge and 10 days postchallenge. Tracheal and cloacal swabs were taken from chickens on days 3 and 7 postchallenge.

Studies of standardized reverse genetics H5N3 vaccine

Experiment 1

Thirty 2-week-old SPF white Leghorn chickens in six groups were immunized subcutaneously at the base of the neck with 0.5 ml of three different inactivated rgH5N3 vaccines, as follows: (1) allantoic fluid virus vaccine, (2) Amicon-concentrated virus vaccine, (3) purified virus vaccine in oil emulsion adjuvant, and (4) normal allantoic fluid

vaccine. Six weeks later, the chickens' sera were tested by HI for immunity to H5N1 virus.

Experiment 2

Forty-five 1-week-old SPF white Leghorn chickens in five groups were vaccinated subcutaneously at the base of the neck with 0.5 ml of one of three different rgH5N3-inactivated viruses. Three weeks later, the chickens' sera were tested by HI for immunity to A/CK/HK/86.3/02 (H5N1) virus. The protective effects of vaccination were evaluated by exposure of the nine vaccinated birds in each group to one chicken that had been intranasally challenged with 100 CLD_{50} of A/CK/HK/86.3/02 (H5N1) virus in 300 μl. On day 3 and day 7 after challenge, tracheal and cloacal swab samples were obtained as previously described (Garcia et al., 1998). Clinical signs were observed for 10 days after challenge.

Experiment 3

Groups of 1-week-old SPF white Leghorn chickens were immunized by subcutaneous injection at the base of the neck with 0.5 ml of allantoic fluid containing inactivated H5N3 viruses in oil emulsion. Three weeks later, the chickens' sera were tested by H1 for immunity to A/CK/HK/86.3/02 (H5N1) virus. The efficacy of the vaccine was evaluated by exposing the vaccinated chickens to birds lethally challenged intranasally with 100 CLD_{50} of A/CK/HK/86.3/02 (H5N1) in 300 μl. On day 3 and day 7 after challenge, tracheal and cloacal samples were obtained; clinical signs were observed for 10 days after challenge.

Serologic tests

Hemagglutination inhibition and neuraminidase inhibition (NI) assays were performed as previously described (Webster et al., 2002a).

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